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SHORT COMMUNICATION

A comparison on DNA methylation levels in bamboo at five developmental stages

LU Yong-quan • WANG Dong-mei • LI Hai-ying • JIA Qing • WU Ze • LU Wen-feng

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Abstract: Bamboo shoots grow quickly through the rapid elongation of internodes. In order to verify whether DNA methylation affects bamboo shoot elongation, we compared DNA methylation levels at five developmental stages of bamboo (*Phyllostachys praecox*), including young bamboo shoot of 40-cm at length (S1), bamboo shoot of 2-cm at length (S2), young leaf (Y1), leaf (Y2), and aging leaf (M), by using amplified fragment length polymorphism (AFLP) based on KpnI/MseI and Acc65I/MseI platforms. The polymorphism ratio of methylated to unmethylated DNA sites in bamboo shoot (S1 and S2) was higher than that in leaf (Y1, Y2 and M). The polymorphism ratio of methylated to unmethylated DNA sites in S2 was highest (32%). Our results suggest that DNA methylation changed greatly at bamboo growing stages, especially in stage of rapid elongation of internodes.

Keywords: methylation; bamboo shoot; *Phyllostachys praecox*; internode elongation

Introduction

Bamboo is one of the most important forest resources on earth. Over 70 genera of bamboo with over 1200 species occur in natural forests. They are mainly distributed in tropical and subtropical regions (Zhu et al. 1994). It is well known that bamboo shoots grow quickly during the rapid internodal elongation period (Li et al. 1997). According to the results in maize, internodal elongation involves a number of steps, including cell division within the intercalary meristem, cell elongation, and lignification (Martin 1988; Morrison et al. 1994). Several genes required for internodal elongation have been identified (Juarez et al. 2004; Zhou et al. 2006). According to our knowledge, there is no mechanism to explain the rapid elongation of internodes in bamboo. Up to now, only some expressed sequence tags (ESTs) related to bamboo internodal elongation have been reported (Zhou et al. 2010).

DNA methylation is the predominant epigenetic modification of DNA, which modifies the information content of DNA and provides information as to where and when the gene should be expressed (Dahl et al. 2003, Chela et al. 1990). In plants and animals, DNA methylation is species-, tissue-, and organelle-specific, which changes with the age and is regulated by hormones (Vanyushin 2006). DNA Methylation plays an important role during the individual development and cell differentiation (Thyagarajan et al. 2009). We wonder whether there is DNA methylation control during bamboo shoots elongation. Our purpose in this paper is to examine this idea by comparing the changes of DNA methylation level during period of internodal elongation of bamboo with other main stages.

We adopted the amplified fragment length polymorphism (AFLP) to analyze the degree of methylation by using isoschizomers that show different sensitivity to cytosine methylation. In order to detect DNA methylation site, two isoschizomer enzymes Acc65I and KpnI are used to digest DNA. They differ in their sensitivity to DNA methylation (Bednarek et al. 2007). Both enzymes recognize the 5' GGTACC 3' site and cut unmethylation DNA. Acc65I is sensitive to CpG methylation. KpnI, however, is insensitive to methylation. We combined both enzymes with the methylation-insensitive MseI. We were able to detect the methylation difference by employing this isoschizomer combination.

Materials and methods

Material selection

Lei bamboo (*Phyllostachys praecox*) is an important and famous species for its high shoot product and delicious taste. Five sam-

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LU Yong-quan (✉)• WANG Dong-mei • LI Hai-ying • JIA Qing
WU Ze • LU Wen-feng
The Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, Lin'an 311300, P. R. China. E-mail: luyongquan@126.com

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ples of Lei bamboov were collected from natural bamboo forests at the same site in Zhejiang Province, China at the same day of April 20th, 2010. Those five samples mimic five major development stages of bamboo. Sample of aging leaf (M) is the mother bamboo at late stage whose leaf is becoming yellow and dying. Other two samples, such as young leaf (Y1) and leaf (Y2), were collected from aging leaf (M) of bamboo at April of 2008 and April of 2009 separately, which represent youth stages. The remaining two samples, S1 (40 cm at length) and S2 (2 cm at length), are bamboo shoots that shoot out from aging leaf at April of 2010. They represent bamboo baby stage and initial stage, respectively.

DNA isolation

The leaf from bamboo or the internodes from bamboo shoot were processed for DNA isolation immediately after collection. Total genomic DNA was isolated from 200 mg of fresh leaf or internodes tissue using the CTAB method (Murray et al. 1980). All reagents used in this study are guaranteed grade.

AFLP Analysis

We followed the standard AFLP procedure as described by Vos et al. (1995). Genomic DNA was separated into two parts, one was digested by enzymes Acc65I/MseI while the other by KpnI/MseI, followed by adaptor ligation, pre-selection and selective amplification steps. All primers used were synthesized by Nanjing Jinsite Biological Engineering & Technology Company in China (Table 1). PCR was performed in a 20-μL reaction system containing 50 ng of template DNA, 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mmol·L^{-1} of $MgCl_2$, 1 unit of Taq polymerase, and 2 μL of 10×PCR reaction buffer. A touchdown-PCR program (Don et al. 1991) was used as follows: 5 min at 95°C, 10 cycles of 30 s at 95°C, 30 s at 58°C with diminishing 0.3°C per cycle, 1 min at 72°C; 20 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and 7 min at 72°C for a final extension. Each of the reactions was tested twice to confirm the observed bands in each sample. PCR products were separated on 6% non-denaturing polyacrylamide gel (80 Volts, 2.5 h). Gels were silver stained following the procedure of Xu et al (2002).

Table 1. Adapter and primer sequences

Adapter	Sequence (5'-3')
Adapters *Acc65* Ⅰ	CTC GTA GCA TGC GTA CA GTA CTG TAC GCA TGC TAC
Adapters *Kpn* Ⅰ	CTC GTA GCA TGC GTA CAG TAC TGT ACG CAT GCT AC
Adapters *Mse* Ⅰ	TAC TCA GGA CTC ATA GAC GAT GAG TCC TGA G
Acc65 Ⅰ/*Kpn* Ⅰ pre-selective primer	CAT GCG TAC AGT ACC A
Mse Ⅰ pre-selective primer	GAT GAG TCC TGA GTA AC
Acc65 Ⅰ / *Kpn* Ⅰ selective primer	CAT GCG TAC AGT ACC A TGC xxx
Mse Ⅰ selective primer	GAT GAG TCC TGA GTA AC xx

Notes: xxx represented any combination of the nucleotides at the primers 3'ends.

PCR results and Data analysis

The AFLP patterns were converted into binomial matrices consisting of either ones or zeros. Only clear and reproducible bands were counted and scored as 1, otherwise as 0. We count it as a locus if there is no less than "1" at a given DNA ladder marker site. Polymorphisms refer to presence and absence of fragments at given loci among samples.

Results and discussion

DNA methylation level at different stages

In total, we identified 250 and 282 AFLP loci, amplified by five primer combinations, in KpnI/MseI and Acc65I/MseI platforms, respectively (Table 2). There is no polymorphism among the former 250 loci yielded by KpnI/MseI, indicating that five samples in this project are from the same clone and they are uniform in DNA sequence. However, of the 282 loci detected by Acc65I/MseI platform, 32 loci showed polymorphisms among 5 samples (Fig. 1). Since no polymorphism in KpnI/MseI platforms among those samples, we can infer logically that the polymorphisms in Acc65I/MseI platform resulted from the difference of DNA methylation at same genomic site among those five samples. In the Acc65I/MseI platform, 20 loci presented fragments in all five samples. Whereas, none of these 20 loci can be detected in KpnI/MseI platform, indicating that the bands at these loci were digested by KpnI/MseI and can not be detected. But owing to sensitive to methylation, methylated bands at these loci can not be digested by Acc65I/MseI, fragments present in five samples. These results show that approximately 7% of the genome are uniformly methylated in stages of M, S1, S2, Y1 and Y2.

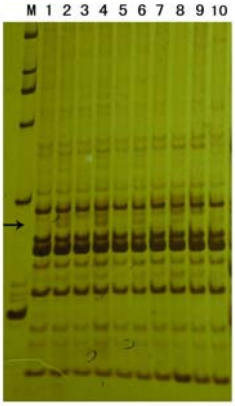

Fig. 1 PCR products screened by non-denaturing polyacrylamide gel (M Land Marker). Every two neighboring Lanes (from 1 to 10) are separated from same samples of Y1 (lanes 1 and 2), S1 (lanes 3 and 4), Y2 (lanes 5 and 6), S2 (lanes 7 and 8) and M (lanes 9 and 10) respectively. Odd Lanes are PCR products with KpnⅠ/MseⅠ platform. Even lanes are PCR products with Acc65Ⅰ/MseⅠ platform. The arrow is the position that displays polymorphism.

Table 2. Loci and polymophisms yielded by KpnI/MseI and Acc65I/MseI

Enzyme Platform	KpnI/MseI	Acc65I/MseI
Total loci	250	282
Polymorphism within platform	0	32
Polymorphism between platforms	--	20

Comparison of DNA methylation levels among different stages

We compared polymorphisms using different enzyme platform in same DNA sample. These polymorphisms indicated that there is the methylation variation in same DNA sample, due to the insensitive/sensitive to methylation of KpnI/Acc65I. We examined the variation of DNA methylation in individual sample and compared the DNA methylation levels. A total of 59 bands showed that methylation polymorphisms were within same sample using different enzyme platform (Table 3). The results of comparison show that the methylation level in bamboo at aging leaf (M) was lowest, and value of polymorphic band was 6. The methylation level in young bamboo shoot (S2) was highest, and value of polymorphic band was 19. The methylation level in young leaf of Y1 and Y2 had no difference, and the value of polymorphic bands was 10. The polymorphism ratio in S2 was the highest (32%) in contrast to the other four samples. This findings support our hypothesis that DNA methylation plays a role in bamboo shoot elongation. Moreover, S1 was elder than S2. The methylation level in S1 exhibits decent, which imply that demethylation exists in the later stage of bamboo shoot growing. These results suggest that DNA methylation might be responsible for fast elongation of internodes in bamboo shoot.

Table 3. Comparison of polymorphic bands among five samples

Samples	Polymorphic band	Percentage
Young leaf ,Y1	10	17%
Young leaf ,Y2	10	17%
Bamboo shoot, S1	14	24%
Bamboo shoot, S2	19	32%
Aging leaf, M	6	10%
Total	59	100%

Discussion

The bamboos in this research are same clone and grow in the same place. They differ only at growth period/age and represent intrinsic developmental stages. Therefore, the results of this research can reflect methylation level changing at different developmental stages. Our results suggest that DNA methylation level changed at bamboo growing stages, especially in stage of rapid elongation of internodes. Evidently, methylation may control the genes expression related to rapid growth of bamboo shoot and internodal elongation.

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